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Biosystematic Study of Japanese *Conocephalum japonicum* (Hepaticae) based on *rbcL* Sequence and Allozyme Data

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In order to examine possible nucleotide sequence variations in the *rbcL* gene of *Conocephalum japonicum,* we collected plant materials from 52 localities representing the geographical distribution of the species in Japan. We determined 1304 nucleotide sequences and found two kinds of *rbcL* sequences differing by 6 nucleotides. Their distribution areas were clearly demarcated as north or south of latitude 42-43° N in Hokkaido. We identified the two *rbcL* types as JN (Japan North) and JS (Japan South) types. The geographical boundary of these two *rbcL* types is nearly identical to the northern limit of the distribution range of *Fagus crenata* in Japan. We could not find any morphological differences between the JN and JS types. In Abuta, Hokkaido, the two *rbcL* types were growing together within small research areas measuring 25 m × 15 m (JN type: 10 and JS type: 8 individuals). We performed an allozyme analysis on this population, and observed tight associations between the allozyme and *rbcL* variations: the JS type had *Est-3a* and *Tpi-1a* alleles, whereas the JN type had *Est-3b* and *Tpi-1b*. These findings suggest that these two *rbcL* types of *C. japonicum* might be two reproductively isolated cryptic species.

Key words: allozyme, bryophytes, *Conocephalum japonicum*, cryptic species, genetic variation, *rbcL*, reproductive isolation

Bryophytes are small land plants with a simple morphology. Most species of bryophytes have been described only by their limited morphological information. Therefore, we can predict the existence of several biologically distinct units or cryptic species, which are genetically and ecologically well-differentiated from each other but morphologically not easily distinguished within the morphological species of bryophytes.

Several cryptic species have been recorded in pteridophytes, which also have a relatively simple morphology among the land plants. Using cytological and allozyme information, cryptic species were recognized from several fern genera (Paris *et al.* 1989). Recently, nucleotide sequencing of the *rbcL* gene was suggested to be useful for recognizing cryptic species of ferns. In various fern species such as *Asplenium nidus*, a large number of variations in *rbcL* sequences have been reported (Murakami *et al.* 1999, Yatabe *et al.* 2001). Considering the slow evolutionary rate of the *rbcL* gene in ferns (Yatabe *et al.* 1999), these results strongly suggest that some morphological species of ferns contain several cryptic species. In fact, Yatabe *et al.* (2001) used artificial crossing experiments to show that the three *rbcL* types of *Asplenium nidus* found in Mt. Halimun National Park, Indonesia are reproductively isolated. Murakami *et al.* (1999) reported that these three *rbcL* types are also ecologically differentiated. Therefore, it was confirmed that *A. nidus* consists of several cryptic species. In similar ways, cryptic species have been discovered in other species of ferns such as *Ceratopteris thalictroides* (Masuyama *et al.* 2002).

Bryophytes are much simpler in morphology than pteridophytes, because the sporophytes of bryophytes do not develop as extensively as those of pteridophytes. Although the gametophytes of some bryophytes have a much more complex morphology than those of ferns, they are still not comparable to the complex morphology of leaves, stems, roots or vascular bundles of fern sporophytes. Therefore, we can expect even more cryptic species in bryophytes. In fact, the existence of cryptic species in bryophytes has been suggested by various allozyme analyses. Szweykowski & Krzakowa (1979) studied allozyme variations of peroxidase, esterase, glutamate oxaloacetate transaminase and glutamate dehydrogenase in 20 Polish populations of *Conocephalum conicum* (Conocephalaceae, Hepaticae) and found 2-3 allozyme types of this species. Szweykowski *et al.* (1981) examined a sympatrical population of the two different allozyme types of *C. conicum*, and reported that no hybrids or recombinants were produced there. Moreover, Odrzykoski (1987) reported that while 12 out of 101 populations in Poland contained both allozyme types, no sporophytes sampled from such sympatrical populations displayed heterozygosity or hybridity. These results strongly suggest the existence of reproductive isolation between the two allozyme types of *C. conicum*.

Odrzykoski & Szweykoski (1991) continued a more intensive allozyme analysis of *C. conicum*. They examined the allozyme variation of 33 enzyme loci for 96 populations throughout the geographical distribution range of the species, and found five discrete groups. The genetic differentiation among these five groups as measured by genetic distance was as large as those commonly reported between different vascular plant species, and much larger than those between conspecific populations. Except for one type, these allozyme types were morphologically indistinguishable. They concluded that these five allozyme groups were biologically independent cryptic species. Similar results were also reported for Japanese *C. conicum* by Akiyama & Hiraoka (1994). They examined the genetic variation of 23 enzyme loci in 17 populations, and found three allozyme types from Japan. Two of the three Japanese allozyme types were shared with the five types recognized by Odrzykoski & Szweykoski (1991). Thus, six allozyme types have been recognized from *C. conicum* so far. Kim *et al.* (2001) determined the nucleotide sequences of the *psbA* gene encoded in cpDNA for the 19 samples of *C. conicum* that were analyzed by Odrzykoski and Akiyama's groups. They reported that the 6 cryptic species recognized through allozyme analyses were also genetically distinguishable by *psbA* gene analysis.

Cryptic species have also been reported in other bryophytes. Two cryptic species were recognized in both *Pellia epiphylla* and *P. endiviifolia* (Pelliaceae, Hepaticae) through allozyme analysis (Zielinski 1987, Szweykowski & Odrzykoski 1990). Analyses of nuclear *tRNA*^{Leu} intergenic sequences confirmed that *P. epiphylla* and *P. endiviifolia* can each be separated into two cryptic species (Fiedorow *et al.* 2001). Dewey (1988, 1989) and Szweykoski & Odrzykoski (1990) reported that *Riccia dictyospora* (Ricciaceae, Hepaticae) and *Aneura pringuis* (Aneuraceae, Hepaticae) consist of three and two cryptic species, respectively, also based on allozyme data.

In this study, we sought to discover cryptic species in the liverwort *Conocephalum japonicum* using *rbcL* sequence variations as a preliminary indicator. In Japan, *Conocephalum japonicum* is a very common annual plant inhabiting a wide range of ecosystems; it grows on moist soil and rocks throughout the lowlands and the mountains (Kitagawa 1982). The genus *Conocephalum* consists of only two species, *C. conicum* and *C. japonicum*. As noted above, in *C. conicum*, a total of 6 allozyme types that are genetically highly differentiated from each other and thus likely to be distinct biological species, have been recognized in terms of their geographical distribution ranges by Odrzykoski & Szweykoski (1991) and Akiyama & Hiraoka (1994). Two different *rbcL* sequences in *C. conicum* have also been reported by Lewis *et al.* (1997) in the course of molecular phylogenetic analysis based on *rbcL* sequencing of 24 representative liverworts. However, they were interested only in the higher level phylogenetic relationships of basal embryophyte lineages and collected *C. conicum* at only two localities in North America as a part of their broad sampling of liverworts. No detailed analyses of the *rbcL* variation in *C. conicum* has ever been performed.

Conocephalum conicum is widely distributed in the Northern hemisphere between the latitudes of 40 and 60° N (Müller 1954); much work would be needed to complete a sufficient analysis of its intraspecific *rbcL* sequence variation. In contrast, the geographical distribution of *C. japonicum* is limited to a much narrower area of East Asia, with Nepal, Japan, Kamchatka (Russia) and Taiwan defining the west, east, north, and south boundaries of its range. We considered it much easier to examine intra-morphospecific *rbcL* variations in *C. japonicum* than in *C. conicum*. We collected *C. japonicum* plant materials from a wide geographical range and from various ecological habitats in Japan, and determined their *rbcL* sequences. We also analyzed some plant samples of *C. conicum* from 3 distant localities for comparison.

In this study we found two *rbcL* sequence types from Japanese *Conocephalum japonicum*. In addition, we investigated the possible existence of reproductive isolation between the two types in a sympatric population using analyses of biparentally inherited allozyme loci in addition to the maternally inherited *rbcL* gene.

Materials and Methods

Plant materials

We collected *Conocephalum japonicum* plant materials from 52 localities across the entire distribution area in Japan (Table 1). We generally collected one individual from each locality. For one population at Abuta-cho, Hokkaido, Japan, we mapped all the recognizable individuals growing in a 25 m × 15 m transect, then collected a small number of plants for the *rbcL* sequencing and enzyme electrophoretic analysis. We also collected samples of *C. conicum* from three distant localities (Table 1) for comparison. All voucher specimens were deposited in the Herbarium of the Graduate School of Science, Kyoto University (KYO).

DNA sequencing and molecular phylogenetic analysis

The total DNAs were extracted using the Nucleon PhytePure plant and fungal DNA extraction kit (Amersham), or alternatively the Plant DNeasy Kit (Qiagen). Partial *rbcL* gene segments were amplified by PCR using Ready-To-Go PCR Beads (Amersham). We used two sets of primers: 1-1 (ATGTCACCACAAACAGAGACTAAAGC), 2R (CTTCTGCTACAAATAAGAATCGATCTCTCCA), N2-1 (TGAAAACGTGAATTCCCAACCGTTTATGCG), NN3-2 (GCAGCAGCTAGTTCCGGGCTCCA) for the PCR (Hasebe *et al.* 1994). A typical PCR amplification included an initial denature (5 min, 94°C) followed by 35 cycles with a 1 min denature at 94°C, 1 min annealing at 50-55°C, 2 min 30 sec synthesis at 72°C, and a final

TABLE 1. The sources Japanese *Conocephalum japonicum* and *C. conicum* sequenced for *rbcL*.

Locality	Voucher
C. japonicum (JN type)	
1. Wakkanai, Hokkaido	HM3024
2. nakagawa, Hokkaido	HM3041
3. Bihoro, Hokkaido	HM3045
4. Teshikaga, Hokkaido	HM3043
5. Kushiro, Hokkaido	HM3042
6. Asahikawa, Hokkaido	HM3040
7. Furano, Hokkaido	HM3026
8. Niikappu, Hokkaido	HM3030
9. Sapporo, Hokkaido	HM1221
10. Otaru, Hokkaido	HM3071
11. Tomakomai, Hokkaido	HM3032
12. Shiraoi, Hokkaido	HM3036
13. Noboribetsu, Hokkaido	HM3038
14. Tomiura, Hokkaido	HM3072
15. Horobetsu, Hokkaido	HM3073
16. Washibetsu, Hokkaido	HM3074
17. Motowanishi, Hokkaido	HM3134
18. Kogane, Hokkaido	HM3076
19. Datemonbetsu, Hokkaido	HM3077
20. Oshamanbe, Hokkaido	HM3044
21. Yakumo, Hokkaido	HM3070
22. Mori, Hokkaido	HM3133
23. Onuma, Hokkaido	HM3035
C. japonicum (JN/JS type)	
24. Abuta, Hokkaido	HM3078Al-18
C. japonicum (JS type)	
25. Muroran, Hokkaido	HM3075
26. Higashimuroran, Hokkaido	HM3031
27. Toyoura, Hokkaido	HM3079
28. Nanae, Hokkaido	HM3080
29. Onakayama, Hokkaido	HM3081
30. Kikyo, Hokkaido	HM3082
31. Goryokaku , Hokkaido	HM3083
32. Hakodate, Hokkaido	HM3023
33. Aomori, Aomori Pref.	HM3021
34. Hirosaki, Aomori Pref.	HM3022
35. Hakkoda, Aomori Pref.	HM3202
36. Yonezawa, Yarnagata Pref.	HM1248
37. Shimogo, Hukushima Pref.	HM1250
38. Mito, Ibaraki Pref.	HM1261
39. Toshima, Tokyo Pref.	HM1256
40. Yokohama , Kanagawa Pref.	HM1254
41. Atami, Shizuoka Pref.	HM1222
42. Minamitsuru, Yamanashi Pref.	HM1265
43. Toyoake, Aichi Pref.	HM1244
44. Gifu, Gifu Pref.	HM1245
45. Kusatsu, Shiga Pref.	HM1263
46. Nagaokakyo, Kyoto Pref.	HM1219
47. Osaka, Osaka Pref.	HM1214
48. Okayama , Okayama Pref.	HM1203
49. Kochi, Kochi Pref.	HM1195
50. Kami , Kochi Pref.	HM1211
51. Beppu , Oita Pref.	HM3141
52. Kanoya , Kagoshima Pref.	HM1260
C. conicum	
53 . Columbia Riv. WA, U.S.A.	HM3068 (DDBJ No AB056154)
54 . Yokohama , Kanagawa Pref., Japan	HM5003 (DDBJ No AB046697)
55. Kushiro, Hokkaido, Japan	HM5012 (DDBJ No AB046696)
56. NC, U.S.A.	(GeneBank No U87066)
57. IL, U.S.A.	(GeneBank No U87067)

step of synthesis for 6 min at 72°C. The PCR products were purified using a QIA quick PCR Purification Kit (Qiagen). The amplified fragments were sequenced with a Big Dye terminator cycle sequencing kit (Applied Biosystems) using the former primers, and run on an Applied Biosystems Model 377 automated DNA sequencer (Applied Biosystems). The obtained sequences were handled by Sequence Navigator software (Applied Biosystems). The molecular phylogenetic tree was constructed using the CLUSTALX program, version 1.8 (Thompson *et al*., 1997) and PAUP program, version 3.1.1 (Swofford 1993).

Allozyme analysis

About 100 mg of fresh leaf tissue was homogenized in a 1 ml extraction buffer (0.2% v/v 2-mercaptoethanol, 2% w/v polyvinyl-pyrrolidone, 0.1 mM Tris-HCl, 1 mM EDTA4Na, 10 mM KCl, 10 mM MgCl2, pH = 7.5). After centrifuging the homogenates at 10,000 rpm for 10 minutes at 4°C, 20 μl of supernatant was used for the electrophoresis for each enzyme. Vertical polyacrylamide slab gel electrophoresis using a discontinuous buffer system (Shiraishi 1988) was conducted for genotyping of all plant samples. Sixteen enzyme systems were tested for activity, and scorable bands were obtained for the following 10 enzymes: fluorescent esterase (EST E.C.: 3.1.1.1), triosephosphate isomerase (TPI E.C.:5.3.1.1), 6-phosphoglconate dehydrogenase (6PG E.C.:1.1.1.44), shikimate dehydrogenase (SKD E.C.:1.1.1.25), glutamate dehydrogenase (GDH E.C.:1.4.1.2), diaphorase (DIA E.C.:1.6.4.3), phosphoglucoisomerase (PGI E.C.:5.3.1.9), glutamate oxaloacetate transaminase (GOT E.C.:2.6.1.1), acid phosphatase (ACP E.C.:3.1.3.2), and superoxidedesmutase (SOD E.C.:1.15.1.1). The loci were numbered in relation to their mobility; the longest distance zone was isozyme 1, and the shorter distance zones were isozymes 2, 3 and so on. The allele labels alphabetically denote relative mobility; the *a*-alleles of each locus have the longest distance, followed by *b*, *c* and so on.

Results

We determined 1304 nucleotide sequences of the *rbcL* gene for all collected samples of *Conocephalum japonicum* and *C. conicum*. Two *rbcL* sequences differing by 6 nucleotides were found in Japanese *C. japonicum* (Fig. 1). The obtained *rbcL* sequence for the three samples of *C. conicum* were different from each other, and also very different from those of *C. japonicum* (Fig. 2). We made a molecular phylogenetic analysis based on the obtained *rbcL* sequences as well as those of other bryophytes from the DNA database. The obtained molecular tree is shown in Fig. 2. The obtained *rbcL* sequences from the samples of *C. japonicum* and *C. conicum* each made a separate clade.

The *rbcL* types determined for the collected samples are shown in Table 1. The geographical distribution of the two *rbcL* types of *Conocephalum japonicum* is shown in Fig. 3. Their distribution areas were clearly separated by the band of latitude 42-43° N through Hokkaido. According to their geographical distribution, we named the two *rbcL* types JN (Japan North) and JS (Japan South) . We could not find any morphological differences between the JN and JS types of *C. japonicum* (Fig. 4). In Abuta, Hokkaido, we found a mixed population of JN and JS types (JN type: 10 and JS type: 8 individuals). In this site, the two *rbcL* types were growing side by side within small areas measuring 25 m × 15 m (Fig. 5). Sporophytes with spores were observed in this population (Fig. 6), suggesting the existence of sexual reproduction , though *C. japonicum* can also propagate asexually through gemma. We performed an allozyme analysis of the 18 samples collected from the Abuta population. Polymorphisms were detected in only two enzymes, EST and TPI. Tight associations between the allozyme and *rbcL* variations were observed: the JS type always displayed *Est-3a* and *Tpi-1a* alleles, whereas the JN type always displayed *Est-3b* and *Tpi-1b* (Fig. 7). Individuals with genotypes of other combinations were not found in this population. The probability for these *rbcL* and allozyme types to exist at once, as observed at the Abuta population, but in the context of cross-breeding, can be calculated using the following formula:

$$\sum_{r=0}^{18} {}_{18}C_r((1/2)^3)^r \cdot ((1/2)^3)^{18-r}$$

The probability was calculated to be less than 1×10^{-10}, which makes a chance occurrence of such an arrangement highly unlikely if all individuals were sexually reproduced.

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JN)  GGATTCAAAG CTGGTGTTAA AGATTATAGA TTAACTTATT ACACTCCGGA TTATGAAACT AAAGAGACAG ATATTTTAGC   80
JS)  ---------- ---------- ---------- ---------- ---------- ---------- -----T---- ----------

JN)  AGCATTTAGA ATGACTCCTC AGCCTGGGGT ACCAGCAGAA GAAGCAGGAG CAGCAGTTGC TGCTGAATCT TCTACCGGTA  160
JS)  ---------- ---------- ---------- -------C-- ---------- ---------- ---------- ----------

JN)  CATGGACTAC AGTTTGGACT GATGGTCTTA CTAATCTTGA TCGTTATAAA GGTCGATGCT ATGGTATTGA CCCTGTTGCT  240
JS)  ---------- ---------- ---------- ---------- ---------- ---------- ---------- -------C--

JN)  GGAGAAGAAA ATCAATATAT TGCTTATGTA GCTTATCCTT TAGATTTATT TGAAGAAGGA TCTGTTACAA ATATGTTTAC  320
JS)  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------

JN)  TTCTATTGTA GGTAATGTAT TTGGATTTAA AGCTTTAAGA GCATTACGTC TTGAAGATTT ACGAATTCCT CCAGCTTATA  400
JS)  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------

JN)  CAAAAACTTT CCAAGGTCCT CCTCATGGTA TTCAAGTTGA AAGAGATAAA TTAAACAAAT ATGGTCGTCC TTTATTAGGA  480
JS)  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------

JN)  TGTACTATTA AACCAAAATT AGGTTTATCT GCTAAAAATT ATGGTAGAGC TGTATATGAA TGTCTTCGTG GTGGACTTGA  560
JS)  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------

JN)  CTTTACTAAA GATGATGAAA ATGTAAATTC TCAACCATTT ATGCGTTGGA GAGATCGTTT CTTATTTGTA GCAGAAGCTC  640
JS)  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------

JN)  TTTATAAATC TCAATCAGAA ACTGGAGAAA TCAAAGGACA TTATTTAAAT GCTACTGCAG GTACATCTGA AGAAATGTTA  720
JS)  ---T------ ---------- ---------- ---------- ---------- ---------- ---------- ----------

JN)  AAAAGAGCAG CATGTGCTAG AGAGTTAGGT GTACCAATTG TTATGCATGA CTATTTAACT GGTGGTTTTA CTGCAAATAC  800
JS)  ---------- ---------- ---------- ---------- ---------- ---------- ---------- --T-------

JN)  TAGTTTATCT CATTATTGCC GTGATAATGG TTTACTTCTT CATATTCACC GTGCAATGCA TGCAGTTATT GATAGACAAA  880
JS)  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------

JN)  AAAATCATGG TATCCATTTC CGTGTATTAG CTAAAGCTTT ACGTTTATCT GGTGGAGACC ATATTCATGC TGGTACTGTT  960
JS)  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------

JN)  GTAGGTAAAC TTGAAGGAGA CCGTCAAGTT ACTTTAGGTT TCGTAGATTT ACTTCGTGAT GATTATATCG AAAAAGATAG 1040
JS)  ---------- ---------- ----G----- ---------- ---------- ---------- ---------- ----------

JN)  AAGTCGTGGT ATTTATTTTA CACAAGATTG GGTTTCTTTA CCAGGTGTTC TACCTGTAGC GTCTGGAGGA ATTCATGTTT 1120
JS)  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------

JN)  GGCATATGCC TGCTTTAACT GAAATTTTTG GAGATGACTC TGTTTTACAA TTTGGTGGTG GAACTTTAGG CCATCCTTGG 1200
JS)  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------

JN)  GGTAACGCAC CTGGTGCAGT TGCTAACCGA GTTGCTTTAG AAGCGTGTGT ACAAGCACGT AATGAAGGTC GTGATCTTGC 1280
JS)  ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------

JN)  TCGTCNAGGT AATGAAATTA TTCG   1304
JS)  ---------- ---------- ----   1304
```

FIG. 1. The two types of nucleotide sequences of the *rbcL* gene from Japanese *Conocephalum japonicum*. The six differentiated positions between the two types are marked by boxes. The sequence data have been deposited in the DDBJ DNA database with the accession number JN type: AB046694 and JS type: AB046695.

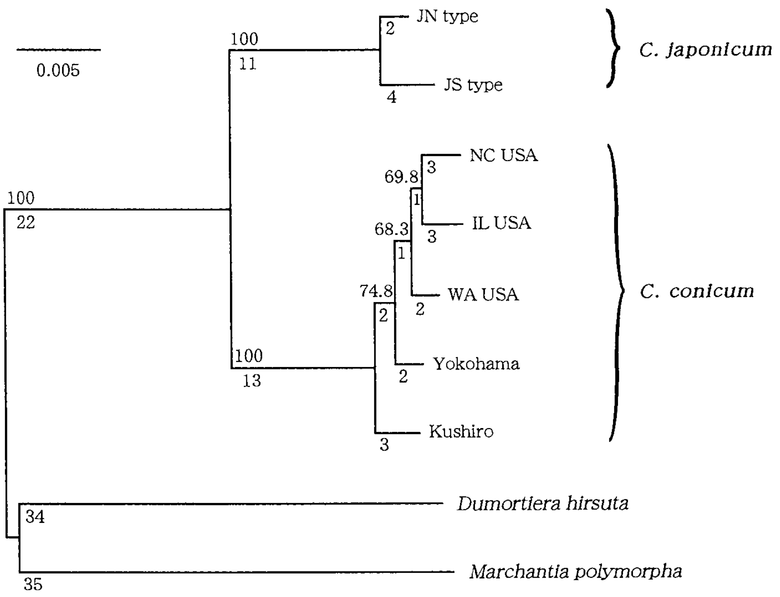

FIG. 2. Neighbor-joining tree based on the nucleotide sequences of the *rbcL* gene. *Marchantia polymorpha* (DNA database accession No X04465) and *Dumortiera hirsuta* (No U87068) were used as outgroups. Numbers above and below the branches are the bootstrap values (%) and numbers of nucleotide substitutions (ACCTRAN optimization), respectively.

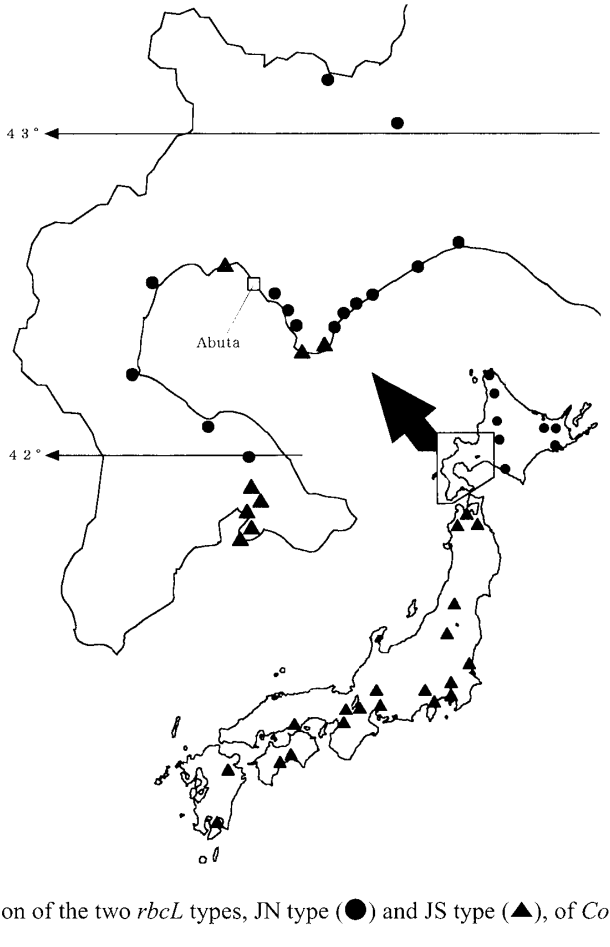

FIG. 3. The geographical distribution of the two *rbcL* types, JN type (●) and JS type (▲), of *Conocephalum japonicum* in Japan.

Discussion

The two *rbcL* types of Japanese *Conocephalum japonicum* found in this study differ by 6 nucleotides. Considering the slow evolutionary rate of the *rbcL* gene, approximately 7-9 × 10^{-11} nucleotide substitutions per site per year in vascular plants (Chase *et al.* 1993, Yatabe *et al.* 1999), these types may be genetically highly differentiated. If the molecular evolutionary rate of the gene in bryophytes is similar, the separation of the two *rbcL* types of *C. japonicum* should go back to approximately 30-36 Ma. These two *rbcL* types might be ecologically differentiated as well, because each *rbcL* type has a distinct geographical distribution. The geographical boundary of these two *rbcL* types of *C. japonicum* is nearly identical to the northern limit of the distribution range of *Fagus crenata* in Japan (Horikawa 1972), part of which is observed in high mountains such as Mt. Hakkoda and Mt. Iwate in Tohoku District. If the geographical boundary of these two *rbcL* types of *C. japonicum* is determined by similar ecological conditions to those of *F. crenata*, we might find substitution from JS type to JN type near the distribution limits of *F. crenata* on those high mountains.

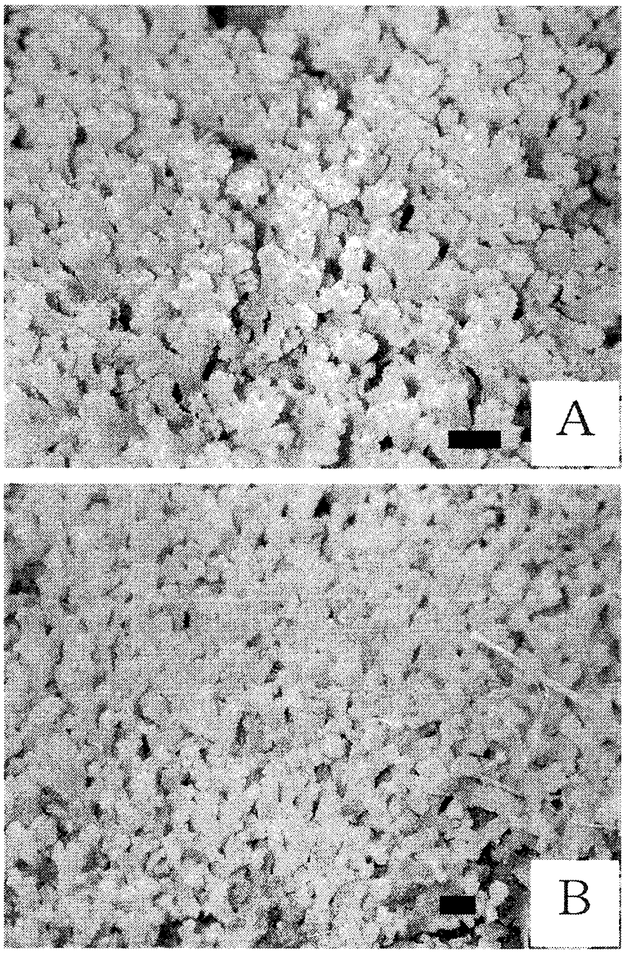

FIG. 4. Morphology of *Conocephalum japonicum*. A, JN type from Abuta, Hokkaido. B, JS type from Yokohama, Kanagawa. Bar: 1 cm.

The geographical boundary between JS and JN types is clear, and sympatrical mixed populations of these two types are very rare. In order to examine the hypothesis that these two *rbcL* types are biologically independent units or cryptic species, it was very important to examine sympatrical populations where the two *rbcL* types were found growing together. Genetic differentiation between the

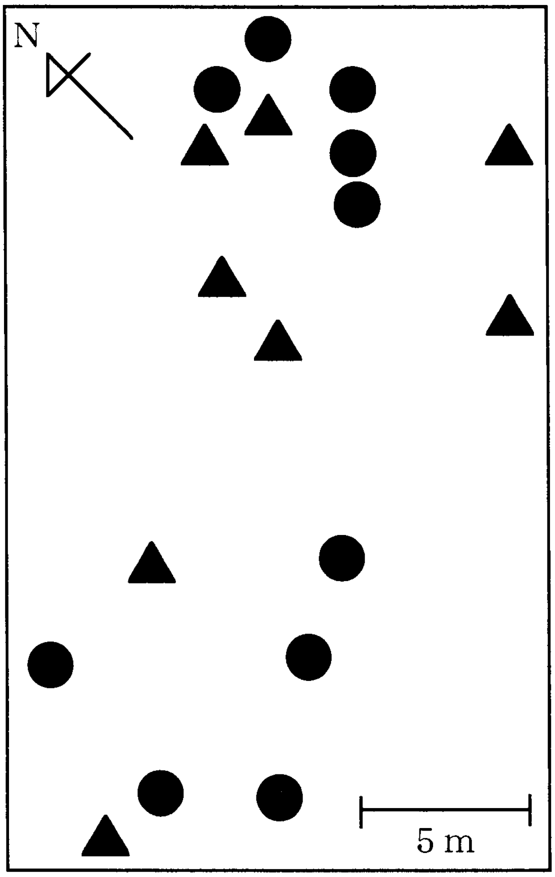

FIG. 5. The map of the two *rbcL* types, JN type (●) and JS type (▲), of *Conocephalum japonicum* in the Abuta population.

two *rbcL* types in these sympatric populations, especially in the several independent nuclear genes, indicates reproductive isolation, which supports their status as different biological species. If the two types were cross-fertile, then genetic analysis should reveal various combinations of *rbcL* types and allozyme alleles. The results of the allozyme analysis that we obtained for the Abuta population showed that the JN and JS types were genetically fixed to different alleles at the *Est-3* and *Tpi-1* loci. This study shows genetic differentiation between the two *rbcL* types in two nuclear genes. It is impossible to completely exclude the possibility that all individuals of the Abuta population were produced through asexual reproduction from two ancestors with two combinations of *rbcL*, *Est-3* and *Tpi-1* genotypes, because *Conocephalum japonicum* produces numerous gemma, and can propagate by an asexual process . However, sporophytes are not rare in various localities of *C. japonicum*, and we actually observed sporophytes in the Abuta population (Fig. 6). Moreover, the same combination of genotypes found in the mixed population at Abuta were also found in geographically distant populations of the JN and JS types. Therefore, we consider it unlikely that all 18 individuals collected in our transect had originated only through gemma. The results of this study strongly suggest the existence of two cryptic species in Japanese *C. japonicum*. In order to confirm the reproductive isolation between JN and JS types, artificial crossing experiments or nuclear genetic variation in each *rbcL* type in the sympatric population must be performed. Itouga *et al.* (1999) collected samples from 14 populations of *C. japonicum* on Honshu, Shikoku and Kyushu Islands, Japan, and two populations in Taiwan. They also intensively examined its genetic variation using allozyme analysis. They reported that *C. japonicum* is genetically an almost uniform species with very few allozyme variations. Its mean genetic diversity within a population proved to be as low as 0.008. However, given the geographical distribution we found, Itouga *et al.* (1999) examined only genetic variation in the JS type because they did not collect samples from Hokkaido. This means that they searched for genetic variation among JS populations and found very little variation in the JS type. If we wish to find nuclear genetic variation in each *rbcL* type for genetic markers, we should examine not the allozyme but DNA level variation. In any case, it will take a long time to clearly confirm the reproductive isolation, because available nuclear DNA markers are rare, and artificial crossing experiments are not easy to perform for bryophytes.

We found two *rbcL* types in Japan, the JN and JS types, which are also genetically differentiated in two allozyme loci. The geographical distribution range of *Conocephalum japonicum* extends to Taiwan, Korea, China, the eastern most part of Russia, and some Eastern Himalayan regions. In the future, we need to analyze samples from these areas in order to determine the geographical distribution range of JN and JS types outside Japan. When we expand the area of investigation, we may also have a chance to find new *rbcL* types of *C. japonicum*, which may prove to be new cryptic species.

It has been shown that *rbcL* sequences can serve as preliminary indicators of cryptic species in various fern groups (Murakami et al. 1998, Yatabe *et al.* 2001). This study suggested that *rbcL* sequences might also be useful in searching for cryptic species of bryophytes. Several cryptic species of bryophytes have been found using allozyme analyses (e.g., Odrzykoski & Szweykoski 1991, Dewey 1989). However, allozyme analyses are applicable only for populations. If two cryptic species are growing sympatrically like the Abuta population, where the JN and JS types were found to be growing together, it may be difficult to find cryptic species using allozyme analyses. In this circumstance, we can expect *rbcL* sequence analysis to be useful because it applies to the individual. Kim *et*

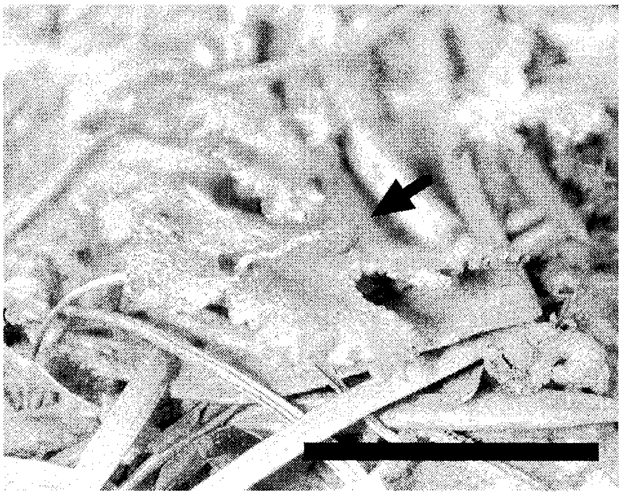

FIG. 6. A sporophyte of *Conocephalum japonicum* found in the Abuta population. Bar: 1 cm.

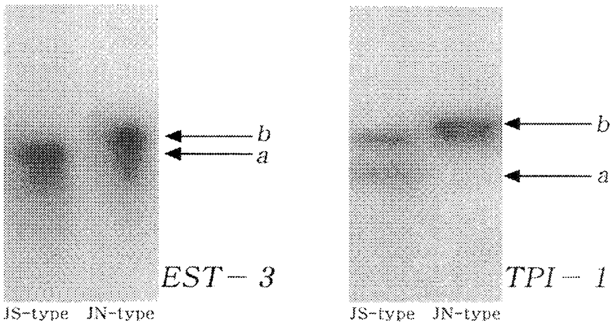

FIG. 7. The allozyme phenograms (EST and TPI) of the JN and JS types of *Conocephalum japonicum*.

al. (2001) used another chloroplast gene, *psbA* for their DNA sequencing analysis of *Conocephalum conicum*. The purpose of their molecular analysis was to clarify the relationship among several possible cryptic species previously recognized in *C. conicum* using allozyme (Odrzykoski & Szweykowski 1991, Akiyama & Hiraoka 1994) and chemical constituent analyses (Toyota *et al.* 1997). Thus, its usefulness in searching for cryptic species of bryophytes is not clear. Presently, PCR amplification and DNA sequence analyses of the *rbcL* gene are much easier than for *psbA* because universal primers have been developed for *rbcL* but not for *psbA*; these primers are applicable to all land plants, from bryophytes to angiosperms (Hasebe *et al.* 1994) . Therefore, at least at this moment we propose that *rbcL* is more suitable than *psbA* as a preliminary indicator in searching for cryptic species of bryophytes.

Several cryptic species have been found in *Conocephalum conicum* using allozyme analysis. Its geographical distribution is very wide, almost throughout the entire Northern hemisphere. If *rbcL* sequence information instead of allozyme variation is used as a preliminary indicator, searching for new cryptic species of *C. conicum* through such a wide distribution area will become more efficient. If sympatric populations of different *rbcL* types of *C. conicum* can be found, a subsequent allozyme analysis such as that conducted on the Abuta population of *C. japonicum* may provide very strong evidence of reproductive isolation. In short, we expect through our studies to establish a new efficient method for species taxonomy of bryophytes.

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